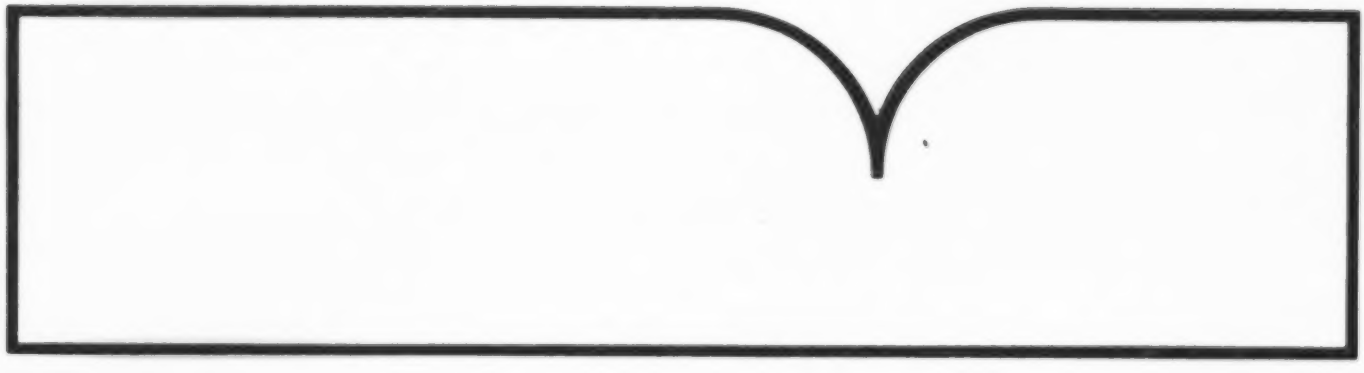
PB87-177119

EPA (Environmental Protection Agency) Developing Methods to Assess Environmental Release

(U.S.) Environmental Protection Agency
Washington, DC

1987



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1. REPORT NO.	2.	3. R PB87-177119
EPA/600/J-87/007		

4. TITLE AND SUBTITLE	5. REPORT DATE
EPA DEVELOPING METHODS TO ASSESS ENVIRONMENTAL RELEASE (Journal Version)	1987
	6. PERFORMING ORGANIZATION CODE EPA/600/16

7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
Levin, Morris, A.; Seidler, Ramon; Bourquin, Al W.; Fowle, John R.; and Barkay, Tammar	

9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
OFFICE OF ENVIRONMENTAL PROCESSES AND EFFECTS RESEARCH OFFICE OF RESEARCH AND DEVELOPMENT U.S. ENVIRONMENTAL PROTECTION AGENCY WASHINGTON, DC 20460	11. CONTRACT/GRANT NO.

12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Same as Item (9)	14. SPONSORING AGENCY CODE EPA/600/16

15. SUPPLEMENTARY NOTES

Bio/Technology, 5:38-45, January 1937

16. ABSTRACT

The rapid advances of biotechnology companies in the areas of pharmaceuticals, energy production, agriculture, industrial chemicals, paper, food preservation, and insect population management have been paralleled by increasing governmental activity. The EPA has invested considerable research effort-intended to meet regulatory needs-toward developing methods for assessing the environmental effects of genetically engineered microorganisms (GEMs).

EPA's biotechnology activities began with a series of three workshops, 1978 - 1980, during which the role of EPA vis-a-vis the potential biotechnology industry was explored. The ORD also surveyed the emerging applications of biotechnology to agriculture and industry, Preliminary investigations centered on the fate, survival, survival, accidental release and potential effects of engineered microbes, as well as the efficacy of biological containment of GEMs in sewage. ORD also identified sepcific Agency capabilities and research needs relative to the regulation of biotechnology products.

The results of these surveys were used to develop a reserch plan. This article describes the surveys, the research plan and progress to date.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Descriptors Biotechnology, Genetic engineering, Risk analysis, Health effects, Environmental effects.		

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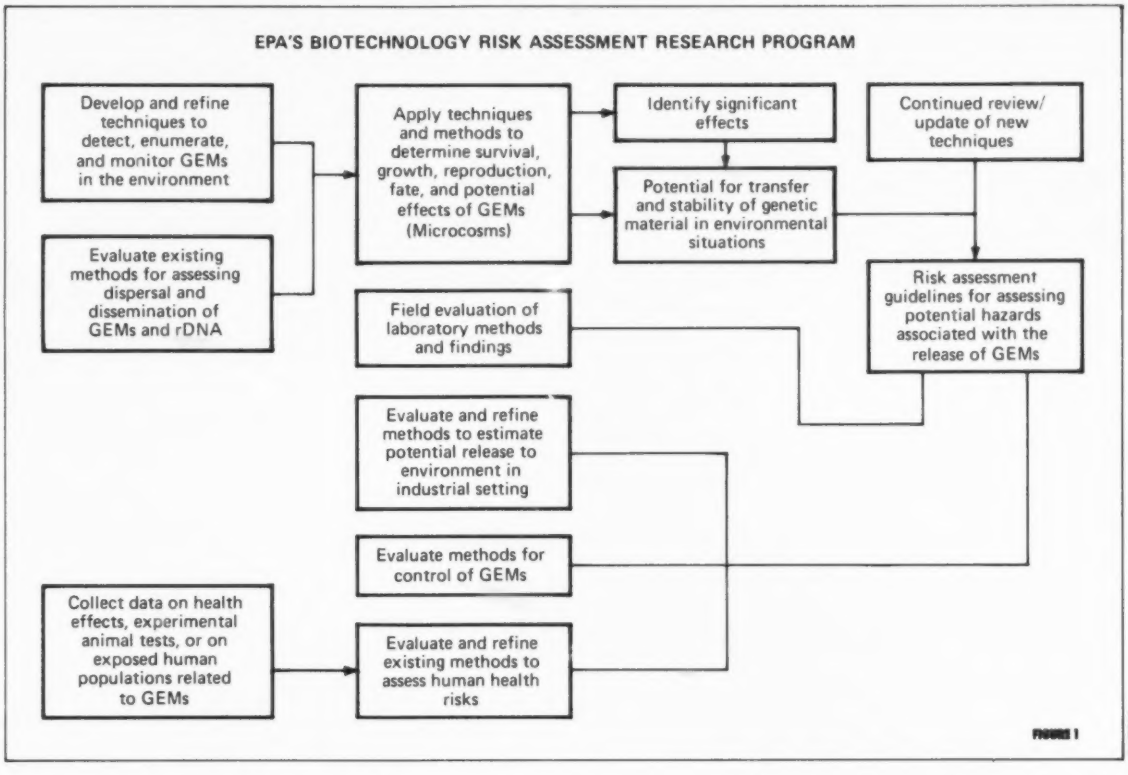
NATIONAL TECHNICAL
INFORMATION SERVICE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
RELEASE TO PUBLIC	UNCLASSIFIED	6
	20. SECURITY CLASS *(This page)* UNCLASSIFIED	22. PRICE

EPA Form 2220-1 (9-73)

EPA/600/J-87/007
JOURNAL ARTICLE

EPA DEVELOPING METHODS TO ASSESS ENVIRONMENTAL RELEASE


EPA'S BIOTECHNOLOGY RISK ASSESSMENT RESEARCH PROGRAM
Develop and refine techniques to detect, enumerate, and monitor GEMs in the environment
Evaluate existing methods for assessing dispersal and dissemination of GEMs and rDNA
Apply techniques and methods to determine survival, growth, reproduction, fate, and potential effects of GEMs (Microcosms)
Identify significant effects
Potential for transfer and stability of genetic material in environmental situations
Continued review/ update of new techniques
Risk assessment guidelines for assessing potential hazards associated with the release of GEMs
Field evaluation of laboratory methods and findings
Evaluate and refine methods to estimate potential release to environment in industrial setting
Evaluate methods for control of GEMs
Collect data on health effects, experimental animal tests, or on exposed human populations related to GEMs
Evaluate and refine existing methods to assess human health risks

The full impact of the applications of genetic engineering to commercial activities has yet to be felt. The rapid advances of biotechnology companies in the areas of pharmaceuticals, energy production, agriculture, industrial chemicals, paper, food preservation, and insect population management have been paralleled by increasing governmental activity. Regulatory agencies and Congressional committees alike are involved in evaluating research, coordination, and regulatory needs.

The Environmental Protection Agency's (EPA) internal structure separates regulatory responsibility from research efforts. The regulatory offices rely on the Office of Research and Development (ORD) to evaluate and develop methods and protocols for assessing potential environmental and human effects of industrial products. ORD research is oriented toward developing new procedures as needed and applying existing knowledge to improve the Agency's analytic capability.

The EPA has invested considerable research effort—intended to meet regulatory needs—toward developing methods for assessing the environmental effects of genetically engineered microorganisms (GEMs).

by Morris A. Levin, Ramon Seidler, Al W. Borquin, John R. Fowle III, and Tamar Barkay, United States Environmental Protection Agency, Washington, D.C. 20460

EPA's Involvement in Biotechnology

EPA's biotechnology activities began with a series of three workshops—held in 1978, 1979, and 1980—during which the role of EPA vis-à-vis the potential biotechnology industry was explored[1]. Industrial, academic, and government scientists reached three major conclusions. The Agency should:

1) Examine sewage systems and aerosols as vectors for the dissemination of genetically engineered microorganisms;

2) Evaluate the potential for adverse environmental effects of engineered organisms; and

3) Develop a research plan for studying the release, survival, colonization, potential for genetic exchange, dissemination, and effects of released engineered organisms.

The ORD proceeded to survey the emerging applications of biotechnology to agriculture and industry. Preliminary investigations centered on the fate, survival, and accidental release of engineered microbes, as well as the efficacy of biological containment of GEMs in sewage. Much of the data from these studies has been published in both government reports and articles[1,2,3] and was summarized at a risk assessment symposium[4].

ORD also began an effort to identify specific Agency capabilities and research needs relative to applying the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Toxic Substances Control Act (TSCA) to biotechnology products. A series of in-house workshops culminated in a conference held at Coolfont, West Virginia (April–May

TABLE 1 Extramural Research Funded by ORD

RECIPIENTS			VISITING SCIENTISTS
Alexander, M., *Cornell University* Fate in natural environments for new genotypes developed by genetic engineering	**Lindow, S., *University of California, Berkeley*** Determination of the fate and effects of released recombinantly generated microorganisms and their parental strains compared with measurements made in contained environments	**Stotzky, G., *New York University*** Fate of genetically engineered microbes in soil and effects on ecological processes	**Chakrabarty, A.** Microbial geneticist, Distinguished visiting scientist
Crawford, D., *University of Idaho* Effects of released recombinant streptomyces on the microbiology and ecological processes of a soil ecosystem	**Miller, R., *Loyola, University of Illinois*** Genetic transfer in aquatic environments	**Suslow, T., *Advanced Genetic Sciences*** Ice nucleation and bioluminescence as sensitive tools for detection of GEMs	**Chatterjee, D.** Microbial geneticist, National Research Council Associate
Colwell, R., *University of Maryland* Survival, modification and effects of genetically engineered microorganisms released to the aquatic environment	**Morita, R., *Oregon State University*** Impact of energy controlled survival on the stability of plasmid DNA in genetically engineered microorganisms	**Tiedje, J., *Michigan State University*** Methods for assessing genetically engineered microbes in soil	**George, E.** Molecular Biology, NRC Associate
Geault, M., *Drexel University* Exchange of recombinant DNA in sewage	**Olson, B., *University of California, Irvine*** Evaluation of novel methods for detection of recombinant DNA in the soil environment	**Van Voris, P., *Battelle Memorial Institute, Northwest*** Evaluation of terrestrial microcosms for assessing fate and effects of genetically engineered microorganisms on ecological processes	**Olsen, R.** Microbial geneticist, Distinguished visiting scientist
Lensky, R., *University of California, Davis* Genetic factors influencing the ecological fate of two model recombinant microorganisms	**Sayler, G., *University of Tennessee*** Genetic approaches for determining persistence and effects of introduced species	**Walter, R., *Florida State University*** The construction of *Bacillus subtilus* strains containing a marker sequence allowing quantitative enumeration in environmental cultures	**Stotzky, G.** Microbial ecologist, Distinguished visiting scientist
	Stahl, D., *University of Illinois* The use of rRNA sequences to characterize natural microbial populations	**Yoder, O. C., *Cornell University*** Effects of foreign genes on virulence of pathogenic fungi	**Walter, D.** Microbiology geneticist, NRC Associate

1984)[5]. The conclusions reached at the conference were used by the Agency to produce an overall research plan and to identify data gaps for future work. The major conference conclusions were:

1) To concentrate on environmental effects, including methodology for estimating fate and effects of engineered microbes; and

2) To adopt a case-by-case approach for evaluating the release of GEMs.

To implement the program, the EPA allocated $1.5 million in FY '85, $4.5 million in FY '86, and $4.8 million in FY '87.

EPA's overall research plan (Fig. 1) focuses on risk assessment and includes development of methods and protocols for laboratory studies, evaluation and modification of methodology in microcosms, evaluation of the use of microcosm data in terms of equivalence to field data, and preparation of risk assessment guidelines.

Several of the Agency's laboratories and a number of universities are involved. The Corvallis, OR, and Gulf Breeze, FL, environmental research labs focus on terrestrial and aquatic ecological assessment problems, respectively. The Health Effects Research Laboratory (Research Triangle Park, NC) focuses on methods to estimate health effects and guidelines for risk assessment. The Cincinnati, OH, laboratory is focused on accidental release and problems associated with containment and decontamination. The Agency has facilities for P-3 level containment and for contained microcosm studies. Table 1 summarizes the range of topics being investigated by extramural researchers funded by ORD.

Eighty percent of biotechnology risk assessment funds are allocated to environmentally oriented research, reflecting the area of deepest uncertainty. Health and industrial issues are closely related to topics covered by the Agency's continuing research on microbial pest control agents (MPCAs) and industrial exposures[5]. Therefore, the following information on the EPA's current research program is biased toward environmental issues.

Detecting, Identifying, and Enumerating GEMs

Adequate assessment of environmental and public health impacts of GEMs requires reliable methodologies for identifying and enumerating microorganisms in complex environmental samples and tissues. The methods must address the analytical and operative criteria required for any monitoring program. That is, they must be sensitive and specific so as to differentiate an extremely low level of GEMs from the complex background of indigenous organisms. The methods must not only be accurate and reproducible, but widely applicable as well. GEMs may be found in samples from ecosystems that differ markedly, such as leaf surfaces and freshwater reservoirs. Fi-

TABLE 2 Methods Being Evaluated/Modified/Tested for the Detection, Identification, and Enumeration of GEMs in Environmental Samples

Method of Detection	Strength	Weakness	Use in Microbial Ecology[1]
Selective Media	High sensitivity	Requires growth on laboratory growth media	Gerhardt, 1981[6]
	Operationally feasible		
Fluorescent Antibody	High specificity	Low sensitivity	Bohlool and Schmidt, 1980[7];
	Direct detection in environmental samples	No differentiation between dead and live cells	Irby et al., 1985[8]; Huang et al., 1985[9]
DNA Gene Probes	Detection is based on the genotype rather than on the phenotype of GEMS	Low sensitivity	Barkay et al., 1985[10]
	Direct detection in environmental samples		
rRNA Sequencing and Finger Printing	High specificity	Low sensitivity	Stahl et al., 1985[11]; Loh et al., 1982[12]; MacDonell and Colwell, 1984[13]
	Very concise	Low operational feasibility	

1. References demonstrate the use of these methods in microbial ecology research.

nally, the methods have to be feasible. They must be cost-effective and technically straightforward. Although some current methods may be directly applicable (Table 2), others will need modification, and all must stand up to comparative evaluation. Each method has its specific strengths and weaknesses, but each must be reliable in its ability to quantitatively enumerate GEMs.

The methods currently under evaluation for detecting, identifying, and enumerating GEMs in environmental samples are summarized briefly below and outlined in Table 2. (For a full description, see "Detection Technology: the Key to Environmental Biotechnology," *Bio/Technology* 4:419, May '86.)

Selective media. This technique is very sensitive, since the medium selects against organisms other than the one of interest. Including more than one selective agent in the medium can increase the precision of detection. The effectiveness of selective media, however, depends on the microorganism's abilities to grow and to express specific genes whose functions are needed for identification. Both of these characteristics may be inhibited by selective agents. In addition, in the natural environment where physiological conditions are less than optimal, some microorganisms enter "nonculturable" stages. DNA elements are lost and the expression of specific gene systems may cease or change dramatically[14].

Fluorescent antibodies. Specific organisms in environmental samples can be detected directly by reacting the samples with fluorescent monoclonal antibodies to purified cell wall components. This method has already been used extensively to differentiate between species of *Rhizobium*[7], to detect aquatic microorganisms in "nonculturable" life stages[14] and to differentiate between Baculovirus species. However, the inability to differentiate between living and non-living microorganisms is a limiting factor. Also, the method relies on microscopic observations. Approaches for modification of this technique include sample concentration procedures[7], flow cytometry[14], and enrichment procedures[15].

Hybridization with DNA gene probes. This method relies on the formation of base pairs between homologous nucleic acid sequences, permitting identification of specific sequences homologous to the labeled DNA probe. The method is used for the detection of specific organisms in environmental samples[10,12] and pathogenic organisms in water systems and food products[16]. Since the method is independent of gene expression, identification is assured even though the selected trait may not be expressed. The present method, however, is not sensitive enough to pick out a few GEMs from the indigenous community. Kuritza and Salyers[17] have concluded that more than five percent of the DNA in a sample must react with the probe before detection is possible.

Ribosomal RNA (rRNA) sequencing and fingerprinting. Ribosomal RNA sequences are personal monograms of bacterial species; they have been used to construct phylogenetic trees[18] and to examine relatedness among *Vibrio cholerae* isolates[13]. Although rRNA sequencing is laborious and test results must be confirmed by other procedures, modification and combination of this method with other detection procedures could provide a highly specific and sensitive methodology.

Data comparability. Because the protocols developed will be used by numerous laboratories and industrial concerns, establishing the comparable reliability and efficiency of the various methods is essential to the success of the program. To this end, a set of "bench mark" reference organisms has been constructed. They are being used by all the laboratories involved in EPA's biotechnology program. The "bench mark" organisms are bacterial strains containing selected plasmids and transposons, developed by Ron Olsen (University of Michigan). In addition to serving as quality assurance measures (i.e., allowing standardization of procedures across laboratories), the reference organisms will help determine which method of detection is most appropriate for a particular GEM and for its environment.

Application of Methodology

As the methods become available, program scientists can begin to address questions pertaining to genetic transfer, stability, and effects on the ecosystem. Ecosystem response to the release of GEMs depends, in part, on the stability of the recombinant DNA (rDNA) in the environment. Even if the gene products are capable of adverse ecological effects, the rDNA's instability or lack of expression in the environment would result in reduced risk. On the other hand, horizontal gene transmission from GEMs to indigenous flora may affect the ecosystem if the exchange results in a more stable inheritance and expression. Critical evaluation of existing data indicates that genetic material is lost, yet also transmitted in the environment[19].

Current studies are listed in Table 1 and described below. These studies include efforts to estimate gene trans-

fer rates in the environment and effects of ecological factors on gene expression and stability, as well as the impact of GEMs on ecological processes.

The significance and frequency of gene transfer in the environment will be evaluated by observing gene transfer frequencies under optimal laboratory conditions, in sterile and nonsterile aquatic and terrestrial systems, in microcosms, and in contained chambers placed in the environment. These studies will also examine the validity of using laboratory experiments to simulate *in situ* transfer events.

The effect of ecological factors on genetic stability will be determined by tracking specific genes while varying parameters known to affect microbial activity (e.g., nutrient concentrations, particulate matter, pH, Eh, temperature, salinity). A comparison of samples from around the world should shed some light on the effect of soil type on detection, survival, and growth of GEMs. Parameters found to affect genetic stability in *in vitro* experiments will then be studied in soil and aquatic microcosms.

The role of position-effect on genetic stability will be determined by following the fate of rDNA inserted in different parts of the microbial genome. The stability of recombinant genes incorporated into plasmids, transposons, and chromosomes will be tested in host systems that are routinely manipulated by the biotechnology industry for environmental application. The location and organization of rDNA in transconjugates (transductants and transformants) will be determined using restriction enzymes, fingerprinting, and Southern hybridization. These studies will identify some of the molecular characteristics that affect stability and spread of rDNA to indigenous flora.

Factors affecting gene expression in the environment will be studied by comparing the genotype with the phenotype of GEMs in microcosms. It is likely that GEMs will be released into environments where their activities are required, and therefore the environment will either naturally support or will be induced to support genetic stability. Research efforts will focus specifically on identifying those factors that may act as positive selectors for the stability and expression of rDNA.

Ultimately, the results of laboratory studies and field validation efforts will be integrated into mathematical models whose validity could be confirmed by field observations. Historically, this approach has been used to predict the environmental fate of chemical pollutants[20]. Existing models for gene transfer among bacterial populations[21] could be modified to describe these events in natural environments.

Health Effects Research

Most of the EPA's biotechnology-oriented health effects research is a direct extension of the 10-year-old Microbial Pesticides Control Agents program, aimed not only at identifying and characterizing these agents, but also at determining their safety vis-à-vis non-target species. From 1974 to 1980, the Health Effects Research Laboratory's efforts were directed toward developing techniques that could be used by pesticide manufacturers and the EPA to identify Baculoviruses.

Early studies focused on characterizing the viruses and developing monitoring methods to detect and identify them. The results demonstrated that Baculovirus species yield characteristic and unique DNA restriction endonuclease fragments and SDS polyacrylamide gel patterns. This provided an identification procedure, so that proteins or DNA from an unknown Baculovirus source can now be compared to a known reference[22,23,24,25]. Confirmatory techniques using DNA hybridization procedures[25] and radioimmunoassays[26] provide supportive identification methods.

There are more complex microbial pest control organisms available—including Bacillus strains, fungi, and protozoans such as the microsporidium *Nosema locustae*. Restriction endonuclease fragment analysis is not as useful for identifying these organisms because of the size and complexity of their genomes. Emphasis has shifted to techniques employing specific antibodies.

Enzyme-linked immunosorbent assays (ELISA) incorporating monoclonal antibodies have been used to characterize and differentiate microsporidian species, as well as the insecticidal and putative hemolytic components of strains of *Bacillus thuringiensis* subspecies *israelensis* and *kurstaki*.

EPA researchers have cloned the genes for several of the *B. thuringiensis* proteins, and are characterizing them in combination with the ELISA studies. It is anticipated that biological activities reported in the literature can be assigned to specific proteins and the specific locations of their genes determined. The role of plasmids in the expression of the genes controlling toxin crystal formation will be examined by transfer of plasmids into non-crystal forming bacteria by conjugation and by transpositional mutagenesis (using a temperature-resistant plasmid).

Information on the ability and rate of genetic transfer as well as the potential for expression of the transferred rDNA will be used to evaluate the potential for adverse mammalian health effects of Baculoviruses and *Bacillus thuringiensis*.

Quantitative procedures are essential for developing an understanding of the mechanisms associated with survival, colonization, and effects of introduced microbes. With environmental information and data from laboratory effects tests, predictive models could become an integral part of the regulatory decision-making procedure.

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